AD NUMBER

AD465197

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AD465197

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 225

AGAR-GEL PRECIPITIN-INHIBITION TECHNIQUE FOR PLAGUE ANTIBODY DETERMINATIONS

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Project 1C622401A072

June 1965

In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

ACKNOWLEDGMENTS

Acknowledgment is made of the technical assistance of Messrs. Paul J. Pristas, Antonio T. Marallo, Maurice M. Mount, Jr., and Carroll Hill. Mr. Robert R. Brubaker kindly supplied the rabbit antiserum to Pasteurella pseudotuberculosis, strain PB1/-, used in the cross-reaction determination. Dr. John White kindly supplied the monkey sera for serological evaluation of plague antibodies and the experimental data that resulted in Table 4. Special acknowledgment is extended to Drs. Michael J. Surgalla and Sidney J. Silverman for their aid in preparation of this manuscript.

ABSTRACT

The agar-gel precipitin-inhibition technique of Ray and Kadull can be used to detect plague antibodies in human and animal sera after a series of plague vaccine inoculations or after exposure to Pasteurella pestis.

Determination of the minimum reacting concentrations of the plague antigen and antibody reagents, methods for combining reagents, and length of incubation periods are discussed.

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I. INTRODUCTION

The agar-gel precipitin-inhibition technique of Ray and Kadull[1] and Ray[2] has been applied to anthrax antibody and C-reactive protein determinations, respectively, in animal and human serum specimens. This test is simply performed, has a high degree of sensitivity, and was reproducible even when employed by several technicians who were unfamiliar with the method.

This test procedure has been modified to assay antibodies for Pasteurella pestis accurately in vaccinated and infected human and animal subjects.

II. MATERIALS AND METHODS

A. PREPARATION OF AGAR DIFFUSION TEST PLATES AND READING LAMP

The medium test plates and agar diffusion reading lamp were prepared as described by Ray and Kadull.[1]

B. PASTEURELLA PESTIS TEST ANTIGEN

The antigen used in this proposed serological test was obtained from outdated whole-cell formalin-killed plague vaccine lots G 1016, G 0990, E 4649, E 9868, E 9383, and E 9135 prepared at Cutter Laboratories.

C. PASTEURELLA PESTIS TEST ANTISERUM

Three rabbits were intracutaneously injected in the nape of the neck with 0.5 ml of plague Cutter vaccine at two-day intervals for six consecutive injections. After two such courses of injections, a mean titer of 1:256 was attained when the sera were titered by this recommended technique.

D. BOX TITRATION OF ANTIGEN AND ANTIBODY SYSTEM

A box titration of plague antigen versus the plague rabbit antiserum was performed in agar diffusion plates. Five serial twofold dilutions of the antigen (0.5 ml) and of the antiserum (0.2 ml) were prepared in physiological saline. To each dilution an equivalent volume of physiological saline was added to give a final dilution range of 1:2 to 1:32.

The antigen dilutions were added sequentially and in duplicate to the two outer rows of wells (approximately 0.07-ml volume per well) so that one row exactly duplicated the opposite row in serial dilutions of antigen. This was performed in five different agar diffusion plates, one for each subsequent antiserum dilution. After incubation of the plates filled with antigen dilution for 6 hours at 37 C in an atmosphere approaching 100% humidity, the plates were removed from the incubator and the water in the center row of reservoirs was aspirated with a pipette. Then the center row of reservoirs (approximately 0.025 ml per reservoir) was filled with one of the prepared serum dilutions; one plate was used for each dilution of antiserum.

These plates were observed with the aid of the reading lamp after incubation for 24 and 48 hours at 23 to 28 C in polyethylene bags.

The end-point determination was that combination of the highest dilution of antigen and antibody that produced a visible line of precipitate lying between the center and outer rows of wells. This initial titration is essential to the establishment of maximum sensitivity and consistent reproducibility of subsequent tests with unknown sera. This end-point reading was considered to represent a minimum reacting dilution, or MRD_a, of antigen, and a minimum reacting dilution, or MRD_s, of antibody. This is illustrated in Table 1, where the MRD_a of antigen was established as a 1:32 dilution, and the MRD_s of antibody as a 1:8 dilution.

TABLE 1. PLAGUE ANTIGEN AGAR-GEL BOX TITRATION[a/]

Rabbit Antiserum Dilution	Antigen Dilution					
	1:2	1:4	1:8	1:16	1:32	1:64
1:1	+	+	+	+	+	+
1:2	+	+	+	+	+	+
1:4	+	+	+	+	+	±
1:8	+	+	+	+	+	-
1:16	+	±	±	-	-	-
1:32	-	-	-	-	-	-

a. MRD_a = 1:32; MRD_s = 1:8.

E. SERUM TITRATIONS

The inhibition, or indirect, method was used in titrating unknown serum specimens. Serial twofold dilutions of 0.2 ml of unknown serum were made in physiological saline. To each dilution, 0.2 ml of a twofold concentration (1:16) of the previously box-titered antigen (1:32) was added; the final mixtures thus contained an antigen MRD_a plus unknown serum dilutions ranging from 1:2 to 1:32. The mixtures were hand-shaken for 30 seconds and incubated in a 37 C water bath for ½ hour to permit antigen-antibody binding.

Outer rows of wells in an agar diffusion petri dish were sequentially filled in duplicate with the incubated antigen-antibody tube mixtures; thus, each well in one outer row contained the same mixture as the corresponding well in the opposite outer row. These plates were then placed in an incubator containing a tray of water and incubated at 37 C for 6 hours. The water condensate was removed from the center reservoirs prior to adding the 1:8 dilution (MRD_s) of known anti-plague rabbit serum as determined by the box titration (Table 1).

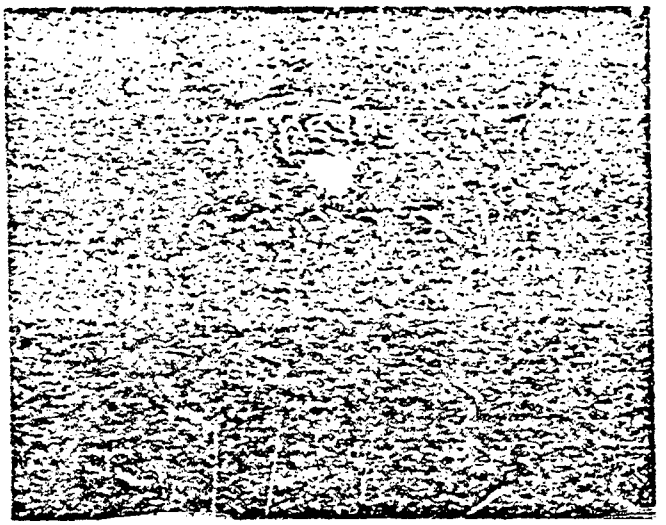
The end-point titer of the unknown serum was determined after incubation in polyethylene bags at 23 to 28 C for 48 hours, with a preliminary reading at 24 hours. The titer was established as that dilution of the unknown serum that completely inhibited the formation of a visible line of precipitate (Figure 1).

Controls consisted of dilutions of the antigen in physiological saline and combinations of the predetermined MRD_a of antigen with known negative and positive anti-plague sera.

III. RESULTS

Test sensitivity was initially determined on sera from plague-vaccinated individuals (Cutter vaccine). Numerous agglutination tests were performed on similar serum specimens without any positive indication of plague antibodies being present.

Three groups of human sera were evaluated: initial vaccine series group, booster vaccine series group, and sera from individuals who had not received any plague vaccine. The booster vaccine series group was further subdivided into a group containing sera from individuals of maximum exposure to P. pestis and a group with minimum exposure to this microorganism (Table 2).



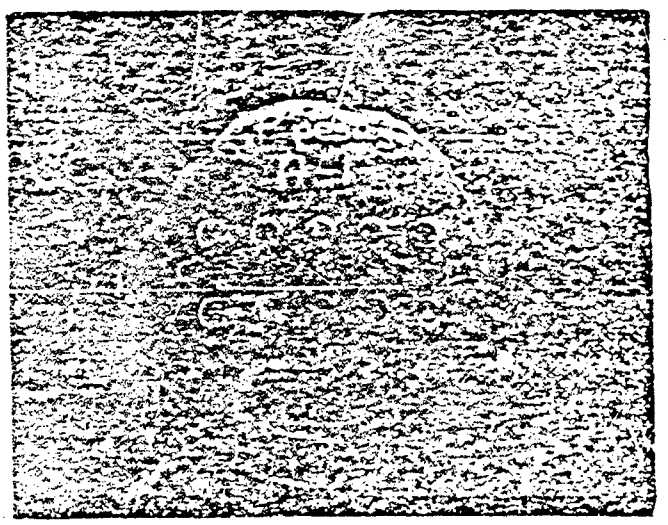


Figure 1. Serum Titrations — Indirect Technique.

A. Negative Serum: Note undulating lines of visible precipitate between center and outer rows of wells, indicating absence of binding of MRD_8.

B. Positive Serum: Note absence of visible line of precipitate between first and second wells on the left of center and outer rows, indicating complete binding of MRD_8; end point is 1:4 dilution of serum.

TABLE 2. AGAR-GEL DIFFUSION COMPARISON OF SERA FROM PLAGUE-VACCINATED (CUTTER) INDIVIDUALS

Vaccine Category	Serum Source	Titer: Direct Method (Double Diffusion)	Titer: Inhibition
None	B.A.	-[b]	-
	W.R.B.	-	-
	H.H.	-	-
	A.L.A.	-	-
	T.B.	-	-
Initial Series	S.S.B.	-	-
	E.A.B.	-	-
Booster Series			
Minimum[a]	I.L.K.	-	1:4
	W.L.K.	-	1:8
	J.S.K.	-	-
	P.M.	-	1:8
	J.P.K.	-	1:2
	D.M.	-	-
Maximum[a]	G.Y.	-	1:8, 1:8
	M.J.S.	-	1:16
	A.A.	-	1:16
	D.M.	-	1:4, 1:2
	C.S.	1:4, 1:2	1:16
	H.T.	1:8, 1:8	1:32
	G.W.	1:8	1:16
	W.D.L.	1:4	1:16
	W.S.D.	-	1:4
	F.D.K.	1:4	1:8, 1:8

a. Possible exposure to organism in laboratory.
b. Negative.

The direct or double diffusion was compared with the inhibition diffusion method in the gel plates. The latter procedure employed the reagents as described above; the direct method employed serial twofold dilutions of the sera in the outer two rows of reservoirs, the center row of reservoirs was filled with the twofold concentration of the plague vaccine antigen (2 MRD_a) or the 1:16 dilution.

The sensitivity of the inhibition technique was demonstrated in the above results when compared with the double diffusion method. Subsequent assay of 299 agglutinin-negative sera from plague-vaccinated individuals showed that 198 were negative, 21 had titers of 1:2, 34 had titers of 1:4, 33 had titers of 1:8, 16 had a 1:16 titer, 5 had titers of 1:32, and two had a 1:64 titer.

Three rabbits were inoculated with a pool of four lots of P. pestis outdated vaccine (Cutter), lots E4649, G 0990, E9868, and G 1016. Each rabbit had a negative prevaccination titer and received two series of six 0.5-ml intramuscular injections in the nape of the neck given every other day for a total of 12 injections. Results of this vaccine series in rabbits are shown in Table 3.

TABLE 3. AGAR-GEL PRECIPITIN-INHIBITION TITERS OF PLAGUE-VACCINATED (CUTTER) RABBITS

Rabbit	Serum Sample	Titer
1	Prevaccinated	negativ
	9 days after Series 1	1:32
	13 days after Series 2	1:128
2	Prevaccinated	negative
	9 days after Series 1	1:8
	13 days after Series 2	1:512
3	Prevaccinated	negative
	9 days after Series 1	1:2
	13 days after Series 2	1:128

A further analysis of anti-plague antibody formation was made in four groups of five monkeys each, which were treated in the following manner. Groups A and B were vaccinated with Cutter Laboratories plague vaccine by 0.5-ml intramuscular injection on days 0, 7, and 14. On day 19 a post-vaccination serum specimen was drawn for its antibody content. Then all groups (A, B, C, and D) were given a live infective dose of P. pestis intraperitoneally on day 21 after initial vaccination. Groups C and D had received no vaccine. Groups A and C monkeys received streptomycin, 15 mg per pound per day every 6 hours for 7 to 10 days, only when the

individual monkey's temperature reached 105 F. Group B and D monkeys received no therapeutic regimen; Group D acted as the control group to the vaccinated, vaccinated-therapy, and therapy groups. All groups had a negative prevaccination titer. The results are presented in Table 4.

Recently an alum-adsorbed plague vaccine was administered to 14 previously immunized individuals. A pre-booster serum was taken from each individual prior to the injection and a post-booster serum was obtained 21 days after the inoculation. Table 5 shows the anti-plague antibody response from a booster injection of plague antigen.

In the above results, a titer increase was demonstrated in vaccinated human sera and in vaccinated and infected monkey sera using the agar-gel precipitin-inhibition serological procedure for P. pestis. Serial serum samples from a laboratory-acquired case of pneumonic plague reported by Burmeister, Tigertt, and Overholt[3] were assayed by this method; the results are shown in Table 6.

IV. DISCUSSION

The serological test procedure represents a [illegible] antibodies by inhibition of a standardized plague antigen-antibody precipitation in agar gel. It attains this sensitivity without the employment of concentrated reactants that are used in the double diffusion technique.

The comparison of the methods in Table 2 indicates that the inhibition method is the more sensitive assay of plague antibodies. However, both methods showed a good degree of reproducibility of titer on the limited duplicated titrations of serum. The difference between the double diffusion method shown in Table 2 and the usual reported double diffusion technique was that the antigen reactant was a dilution ($2\ MRD_a$) of the antigen as determined by the box titration of Table 1. In using such a dilution one obtains a more sensitive diffusible antigen concentration as shown by Ray[2] and there is less chance for nonspecific antibody reactants in serum samples to cause a false positive reaction.

Table 2 demonstrated further that the agar-gel precipitin-inhibition method is able to detect plague antibodies in sera from plague-vaccinated individuals, although these titers are evident only after booster injections. When the plague vaccine was administered to rabbits, it stimulated formation of antibodies that were detected by this method (Table 3); this further substantiates the sensitivity of this method.

TABLE 4 EFFECT OF PLAGUE VACCINE AND THERAPY ON MONKEYS CHALLENGED INTRAPERITONEALLY WITH P. PESTIS

Group	Monkey	Serum Titer by AGPT[a] Prevac.	Serum Titer by AGPT[a] Postvac.	High Temp F	Therapy,[b] Post-challenge, hr	Postmortem Post-challenge, hr	Postmortem Titer	Titer, postchallenge 21 days
A Vaccine + Therapy	5	-[c]	1:4	104		87	-	
	11	-	1:16	105	28			1:16
	12	-	1:8	105	24			1:16
	15	-	1:2	105	84			1:16
	17	-	1:16	104		84	-	
B Vaccine only	4	-	1:32					1:256
	8	-	1:16	104	none	117	-	
	13	-	1:8	105	none	117	-	
	14	-	1:16	105.6	none	132	1:16	
	20	-	1:8	104.2	none	162	-	
C Therapy only	1	-	none	105	24			1:16
	2	-	none	105	84			1:128
	3	-	none	105	66			1:512
	10	-	none	105	60			1:512
	16	-	none	105	86			1:32
D Control	6	-	none	Spiked Temp.	none	All died 72-105 hr.	-	
	7		none	Spiked Temp.	none	All died 72-105 hr.	-	
	9		none	Spiked Temp.	none	All died 72-105 hr.	-	
	18		none	Spiked Temp.	none	All died 72-105 hr.	-	
	19		none	Spiked Temp.	none	All died 75-105 hr.	-	

a. Agar-gel precipitin inhibition.
b. Streptomycin - 15 mg per pound per day; every 6 hours for 7 to 10 days when temperature reached 105 F.
c. Negative.

TABLE 5. AGAR-GEL PRECIPITIN-INHIBITION TITERS OF PRE- AND POST-BOOSTER SERA; EVALUATION OF ALUM-COATED PLAGUE VACCINE

Serum Code	Pre-Booster Titer	21-Day Post-Booster Titer
Bea, A.	-a/	1:8
Bel, A.	-	1:32
Boy, W.	-	1:16
Dec, J.	-	1:32
Dra, E.	-	Negative
Eng, D.	-	1:1024
Gou, R.	-	1:32
Gro, G.	-	1:16
Har, C.	1:16	1:64
Lan, D.	1:16	1:16
Nor, E.	-	1:32
Pow, R.	1:4	1:64
Ram, O.	-	1:16
Swe, C.	-	1:16

a. Negative

TABLE 6. AGAR-GEL PRECIPITIN-INHIBITION TITERS OF SERIAL SERUM SAMPLES FROM A CASE OF PNEUMONIC PLAGUE

Serum Specimen Date	Days After Hospitalization	AGPI Titer
August 10	-22	negative
September 2	2	negative
7	7	1:4
9	9	1:32
11	11	1:32
14	14	1:64
18	18 (discharged)	1:64
28	28	1:64
30	30	1:16
October 2	32	1:32
12	42	1:16
19	49	1:32
November 3	64	1:32
January 6	128	1:16
May 16	258	1:4
July 15	318	1:4
August 22	356	negative

In determining the effect of vaccination and therapy in monkeys that were injected by the intraperitoneal route with virulent P. pestis (Table 4) the results show that there was an increase in plague antibodies due to vaccine as well as from the live injected plague bacillus. However, only one monkey in Group B, which had an immune vaccine response, survived the intraperitoneal challenge and this monkey had the highest titer prior to challenge.

These data (Table 4) indicate that the measured antibody titer of 1:2 through 1:16 was overwhelmed by the inoculated plague challenge dose. The vaccine apparently forestalled death in these monkeys in comparison with the postchallenge time to death of the other monkey groups. Although those Group B monkeys took longer to die, indicating some modification of the disease due to the vaccine, streptomycin therapy when given early in the disease proved better than either the vaccine alone or vaccine in combination with streptomycin therapy. Table 4 further indicates that either the vaccine does not confer immunity or not enough vaccine was given to protect the monkeys under conditions of the test.

When an experimental alum-adsorbed plague vaccine was administered as a booster injection (Table 5), the 21-day post-booster titer was significantly higher than those obtained in the human (Table 2) or rabbit whole-cell formalin-killed vaccine studies (Table 3).

Of more importance is the attained titer response of a human laboratory-acquired case (R.L.P.) of plague, as demonstrated in Table 6. This case is closely analogous to the Group A monkey experiment of Table 4, with the exception that R.L.P. had a booster injection of plague vaccine (Cutter) just prior to his exposure. Both R.L.P. and the Group A monkeys received immediate therapy upon developing a high temperature. The recommended test procedure adequately assayed the plague-antibody content of serial serum samples in this human case and was able to detect a rise in plague titer after only 7 days of hospitalization or approximately 12 days after the exposure to the plague bacillus.

A Pasteurella pseudotuberculosis, strain PB1/-, prepared rabbit antiserum failed to inhibit the standardized plague antigen-antibody precipitation in agar gel. This indicated that no cross reaction occurred between this pseudotuberculosis antiserum and the plague antigen used in this serological test procedure. Although P. pseudotuberculosis and P. pestis have many antigens in common, this reaction further supports the findings of Burrows and Bacon that laboratory strains of P. pseudotuberculosis that lack the V and W antigens, as does strain PB1/-, differ from the fully virulent P. pestis strains. However, additional VW-negative and VW-positive P. pseudotuberculosis antisera should be assayed for further substantiation of this reaction.

The recommended test procedure of agar-gel precipitin inhibition sensitively assays antibodies against the plague bacillus in vaccinated and infected human and animal serum samples.

This method does not require the use of concentrated reactants, the addition of a third entity such as complement, latex, or dye particles, nor the use of an indicator system such as chicken, goose, or sensitized sheep red blood cells.

The test method's sensitivity is attained by the inhibition of a known soluble antigen antibody plague precipitin reaction in agar-gel.

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Unclassified
Security Classification

DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)

U.S. Army Biological Laboratories
Fort Detrick, Frederick, Maryland, 21701

2a. REPORT SECURITY CLASSIFICATION

Unclassified

2b. GROUP

3. REPORT TITLE

AGAR-GEL PRECIPITIN-INHIBITION TECHNIQUE FOR PLAGUE ANTIBODY

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

5. AUTHOR(S) (Last name, first name, initial)

Ray, John G., Jr.
Kadull, Paul J.

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
June 1965	20	4

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. 1C622401A072	Technical Manuscript 225
	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)

10. AVAILABILITY/LIMITATION NOTICES

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	U.S. Army Biological Laboratories Fort Detrick, Frederick, Maryland, 21701

13. ABSTRACT

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DD FORM 1 JAN 64 1473

Unclassified
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